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METHOD AND PRODUCT FOR TREATING CANCER IN PETS

FIELD OF THE INVENTION

The present invention relates to a method for treating disease in pets, and more particularly for treating cancer in dogs and cats through addition of vitamin D or an analog of vitamin D to pet food, and to pet food containing vitamin D or a vitamin D analog.

BACKGROUND OF THE INVENTION

Pets play an important role in many peoples lives, and consequently many pet owners will go to considerable lengths to treat their pets for major illnesses, such as cancer. Cancer is one of the major forms of mortality in pets such as cats and dogs, and therefore the pet owners desire ways of treating this disease in their pets to increase their longevity. Such treatments ideally would be not only economical, but also practical for owners rather than veterinarians to administer to the pet.

Present methods of treating cancer in pets focus primarily on surgical resection of solid tumors. Surgery is expensive, and moreover, is not suitable treatment for many cancers. Among these are leukemias and lymphomas, where surgery obviously is not an option, but this class includes highly disseminated malignancies as well as ones with poorly defined margins or those arising in inoperable locations.

It would therefore be desirable to have a way of treating cancer in dogs and other pets that could be administered routinely by pet owners and that would not be resisted by the pet. Ideally, such treatment could be administered with the pet's food.

SUMMARY OF THE INVENTION

The present invention meets these needs by providing a pet food that contains vitamin D or a derivative of vitamin D, hereinafter referred to generically as "vitamin D analogs", where the term "vitamin D analogs" specifically includes 1,25-$(OH)_2$ D_3, analog V, and EB 1089, the structures of which appear in Figure 1. The present invention further provides for a method of treating cancer in a pet, such as a

cat or dog, through feeding the animal a pet food containing at least one vitamin D analog.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Figure 1 shows the chemical structures of vitamin D_3, 1,25-$(OH)_2D_3$, EB 1089, and analog V;

Figure 2 is a microphotograph (X 300) of SCC 2/88 cells immunohistochemically stained with monoclonal anti-vitamin D receptor-antibody, after treatment with 1,25 $(OH)D_3$ (left) or with vehicle (right);

Figure 3 is a microphotograph (X 300) of SCC 2/88 cells immunohistochemically stained with (left) rabbit anti-human parathyroid hormone-related protein (PTHrP), and (right) with non-specific antiserum;

Figure 4 is a histogram showing the growth of SCC 2/88 cells after addition of different concentrations of 1,25$(OH)_2D_3$ and its analogs to the culture medium;

Figure 5 is a phase contrast microphotograph (X 100) showing the morphology of SCC 2/88 cells grown in 6-well plates and treated with (A) vehicle, (B) 1,25-$(OH)_2D_3$; (C) EB 1089; and (D) analog V;

Figure 6 is a phase contrast microphotograph (X 100) showing the morphology of SCC 2/88 cells grown in 6-well plates on day 3 in the absence of a vitamin D analog.

Figure 7 is a histogram showing PTHrP production as measured by release of PTHrP (pg) per DNA (μg) in SCC 2/88 cells treated with vitamin D analogs compared to the vehicle-treated control;

Figure 8 shows a Northern blot analysis of PTHrP mRNA expression in SCC 2/88 cells treated with vehicle, 1,25$(OH)_2D_3$, TGF-β, 1,25$(OH)_2D_3$ and TGF-β, anti-TGF-β, 1,25$(OH)_2D_3$ and anti-TGF-β;

Figure 9 is a histogram showing PTHrP mRNA expression in the 10^{-7} M $1,25(OH)_2D_3$-treated cells at 24 hr and in the 10^{-7} M $1,25(OH)_2D_3$ and TGF-β (1.5 ng/mL)-treated cells at 3 and 6 hr compared to vehicle-treated control;

Figure 10 is a time course Northern blot analysis of TGF-β mRNA expression in SCC 2/88 cells treated with vehicle or $1,25(OH)_2D_3$;

Figure 11 shows a SDS-PAGE and Western blot analysis for involucrin in SCC 2/88 cells treated with EB 1089 (10^{-7} M) and analog V (10^{-7} M and 10^{-9} M).

DETAILED DESCRIPTION OF THE INVENTION

Vitamin D displays a wide range of physiological activities, including stimulation of the immune system, mobilization of calcium from the skeletal system, and cell differentiation, that have suggested its use for treating hypertension, diabetes mellitus, autoimmune diseases, AIDS, host versus graft reactions, and even strengthening egg shells.

Of particular relevance to the present invention, $1,25(OH)_2D_3$ also stimulates differentiation of cells and inhibits excessive cell proliferation such as occurs in cancer. U.S. Pat. No. 4,391,802 issued to Suda et al. discloses that 1α-hydroxyvitamin D compounds induce differentiation of leukemia cells to nonmalignant macrophages (monocytes), and are useful in the treatment of leukemia in humans. In another example, Skowronski et al. reported anti-proliferative and differentiating actions of vitamin D_3 analogs on cell lines derived from human prostate cancers (Skowronski *et al* 1995).

In four thyroid anaplastic carcinoma cell lines, $1,25\text{-}(OH)_2D_3$ caused diphasic cell growth in three of the four cell lines, while the vitamin D analog 22-oxacalcitriol showed dose-dependent inhibition of cell growth in all four of the cell lines. (Suzuki *et al* 1999) $1,25\text{-}(OH)_2D_3$ has anti-proliferative activity in some human and rat liver cancer cell lines, but other cell lines resist its action. (Pourgholami *et al* 2000).

$1,25\text{-}(OH)_2D_3$ also inhibits cell growth and promotes differentiation in a dose-dependent manner in a human prostate cancer cell line ((Moffatt *et al* 1999).

1,25-$(OH)_2D_3$ has significant antitumor effects in the murine squamous cell carcinoma (SCC) tumor model in vitro and in vivo (Hershberger *et al* 1999).

In addition to the antiproliferative effect of 1,25$(OH)_2D_3$ on tumor cells, 1,25$(OH)_2D_3$ and its analogs stimulates differentiation in squamous cell carcinoma (McElwain *et al* 1995), (Kornfehl *et al* 1996), (Yu *et al* 1995), (Hershberger *et al* 1999).

In canine-derived cell lines, treatment of four osteosarcoma cell lines with 1,25$(OH)_2D_3$ increases alkaline phosphatase activity in one cell line, osteocalcin production in two lines and type I collagen production in three lines (Nozaki *et al* 1999). In a canine squamous carcinoma cell line (SCC 2/88) 1,25-$(OH)_2D_3$ stimulates production of parathyroid hormone-related protein (PTHrP), a major causative factor in humoral hypercalcemia of malignancy (Merryman *et al* 1993).

The applicants have found that 1,25$(OH)_2D_3$, 22,24-diene-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3 (EB 1089) and 1,25-dihydroxy-16-ene-23-yne-vitamin D (analog V) inhibit cell proliferation in vitro in the canine-derived SCC 2/88 cell line at a concentration of 10^{-7} M, while EB 1089 inhibits cell growth significantly at concentrations of 10^{-7} M and 10^{-9} M (on three-day treatment).

Figure 1 shows the chemical structures of vitamin D_3, 1,25-$(OH)_2D_3$, 1a,25-$(OH)_2$-16-ene-23-yne- vitamin D (analog V), and 1a,25-dihydroxy-22,24-diene-24,26,27-trihomo vitamin D (EB 1089).

Figure 2 is a microphotograph (X 300) of SCC 2/88 cells immunohistochemically stained with vitamin D receptor-antibody (left) and treated with non-specific antiserum. The left hand side of Figure 2 shows cellular expression of the vitamin D receptor, showing positive labeling in all nuclei of tumor cells (arrowheads). The positive peroxidase reaction in nuclei of carcinoma cells establishes that these cells derived from canine squamous cell carcinoma have receptors for vitamin D. The control section (right) after reaction with non-specific antiserum in place of a specific primary antibody shows the absence of reaction product in the cell nuclei (arrowheads).

Figure 3 is a microphotograph (X 300) of SCC 2/88 cells immunohistochemically stained with (left) rabbit anti-human parathyroid hormone-related protein (PTHrP), showing positive reaction for PTHrP in cell cytoplasm (arrowheads), and (right) SCC 2/88 cells after reaction with non-specific antiserum in place of a specific primary antibody, showing the absence of reaction product in the cell cytoplasm.

Figure 4 is a histogram showing the effect of different concentrations of $1,25(OH)_2D_3$ and its analogs on the growth of SCC 2/88 cells. Addition of a vitamin D analog to the culture medium inhibited cell growth in a dose-dependent manner. Growth of SCC 2/88 cells significantly reduced the concentration of DNA ($\mu g/\mu l$) at 10^{-7} M of $1,25(OH)_2D_3$ ($p<0.01$), EB 1089 ($p<0.001$), and analog V ($p<0.001$) and at 10^{-9} M of EB 1089 ($p<0.05$).

Figure 5 and Figure 6 are phase contrast microphotographs (X 100) of SCC 2/88 cells on day 3 in the presence and absence (respectively) of $1,25(OH)_2D_3$ and its analogs at concentrations of 10^{-7} M and 10^{-9} M, showing that no significant differences in cell morphology were apparent.

Figure 7 is a histogram showing PTHrP production as measured by release of PTHrP (pg) per DNA (μg). Levels of PTHrP (pg) / DNA (μg) by day 3 significantly increased in all three substrate-treated groups ($p<0.05$) treated with 10^{-7} M vitamin D analog compared to the vehicle-treated control. At 10^{-9} M concentration no vitamin D analog produced a significant difference in PTHrP production, as measured by PTHrP (pg)/ DNA (μg).

SCC 2/88 cells constitutively produce PTHrP, which is associated with humoral hypercalcemia of malignancy, at a level that depends on the duration of culture and confluence of cells (Werkmeister *et al* 1993). $1,25(OH)_2D_3$, EB 1089, and analog V promote PTHrP production in the canine SCC 2/88 cell line (Merryman *et al* 1993). On the contrary, in human squamous cell lines, $1,25(OH)_2D_3$ inhibits PTHrP production, suppresses PTHrP gene transcription, and prevents development of the humoral hypercalcemia of malignancy syndrome (Yu *et al* 1995), (Falzon 1997), (Abe *et al* 1998,El Abdaimi *et al* 1999). The effects of vitamin D analogs on cells of different species are, therefore, unpredictable.

$1,25(OH)_2D_3$ was used to investigate the expression of PTHrP mRNA. Transforming growth factors (TGF-β, TGF-α) and interleukin-1 (IL-1) are coproduced with PTHrP in humoral hypercalcemia of malignancy. Particularly, TGF-β copurified with PTHrP from many human and animal cancer-associated with humoral hypercalcemia of malignancy (Merryman *et al* 1994), (Insogna *et al* 1987). Thus, we also compared the effects of TGF-β and anti-TGF-β on PTHrP mRNA expression with the biologically active vitamin D, $1,25(OH)_2D_3$.

In SCC 2/88 cells, TGF-β increases PTHrP production via up-regulating in an autocrine manner, which aggravates the severity of the hypercalcemia (Merryman *et al* 1993), (Merryman *et al* 1994). Correspondingly, levels of PTHrP mRNA in SCC2/88 cells treated with TGF-β increase 2- to 20-fold after 24 hr compared with the vehicle-treated control, in contrast to cells treated with anti-TGF-β. Levels of PTHrP mRNA in cells treated with $1,25(OH)_2D_3$ and TGF-β, however, increased less than in cells treated with TGF-β alone. Cells treated with $1,25(OH)_2D_3$ and TGF-β show 1- to 3-fold higher PTHrP mRNA levels than cells treated with $1,25(OH)_2D_3$ alone. Levels of TGF-β mRNA between $1,25(OH)_2D_3$-treated group and vehicle-treated control did not differ. $1,25(OH)_2D_3$ and TGF-β may therefore upregulate PTHrP production and mRNA expression in SCC 2/88 cells in part due to increased gene transcription. This was most evident at 6 to 12 hr post-treatment. Furthermore, $1,25(OH)_2D_3$ probably affects TGF-β by reducing PTHrP mRNA expression, but not directly decreasing TGF-β mRNA expression.

Figure 8 shows a Northern blot analysis of PTHrP mRNA expression in SCC 2/88 cells treated with vehicle, $1,25(OH)_2D_3$, TGF-β, $1,25(OH)_2D_3$ and TGF-β, anti-TGF-β, $1,25(OH)_2D_3$ and anti-TGF-β. PTHrP mRNA was detectable at all time points (0, 3, 6, 12 and 24 hrs.). All of the lanes were standardized with the glyceraldehyde 3-phosphate dehydrogenase mRNA loading control.

Figure 9 is a histogram showing approximately 1- to 2-fold increases in PTHrP mRNA in the 10^{-7} M $1,25(OH)_2D_3$-treated cells at 24 hr and in the 10^{-7} M $1,25(OH)_2D_3$ and TGF-β (1.5 ng/mL)-treated cells at 3 and 6 hr compared to vehicle-treated control. Levels of PTHrP mRNA in cells treated with TGF-β (1.5 ng/mL) showed a steeper increase of 5- to 20-fold at 6, 12, and 24 hr compared with the

vehicle-treated control. Similarly, the levels of PTHrP mRNA in 10^{-7} M $1,25(OH)_2D_3$ and TGF-β-treated cells (1.5 ng/mL) displayed a 10- to 15-fold greater increase at 12 and 24 hr respectively compared with the vehicle-treated control. Conversely, cells treated with anti-TGF-β (5 μg/mL) or with a combination of $1,25(OH)_2D_3$ (10^{-7} M) and anti-TGF-β (5 μg/mL) showed modest decreases in PTHrP mRNA expression at 24 hr compared to the vehicle-treated control.

Figure 10 is a Northern blot analysis of TGF-β mRNA expression in SCC 2/88 cells treated with vehicle and $1,25(OH)_2D_3$. TGF-β mRNA was detectable at all time points (0, 3, 6, 12 and 24 hrs.), and expression in 10^{-7} M $1,25(OH)_2D_3$-treated cells did not differ significantly from that in the vehicle-treated control cells.

Figure 11 shows a SDS-PAGE and Western blot analysis for involucrin in SCC 2/88 cells treated with EB 1089 (10^{-7} M) and analog V (10^{-7} M and 10^{-9} M). Cells treated with either with EB 1089 or with analog V (at 10^{-7} M in each case) gave bands on the nitrocellulose sheet (molecular weight ca. 66 kDa) that bound a mouse monoclonal antibody against involucrin. Anti-involucrin reactive bands for both EB 1089 (10^{-7} M) and analog V (10^{-7} M) were more weakly defined than that of the vehicle-treated control. Anti-involucrin reactive bands of cells treated with 10^{-9} M analog V were more intense than those of the other treated groups.

Vitamin D analogs inhibit cell growth, as measured by involucrin determinations. Involucrin, a precursor of epidermal cornified envelope, is a marker for squamous epithelium and of terminal differentiation such that decreases in involucrin expression indicate increased cell differentiation. Treatment of post-confluent SCC 2/88 cells with $1,25(OH)_2D_3$, EB 1089, and analog V (at 10^{-7} M) and $1,25(OH)_2D_3$ and EB 1089 (at 10^{-9} M) for up to seven days diminished involucrin expression.

Treatment of cells with 10^{-7} M EB 1089 and analog V yielded weak or substantially absent anti-involucrin reactive bands, compared to cells treated with vehicle alone. Treatment of cells with 10^{-9} M EB 1089 also showed diminished levels of involucrin by Western blot analysis. Furthermore, treatment with EB 1089 or analog V (each at 10^{-7} M) significantly diminished cell growth ($p<0.001$); treatment with EB 1089 (10^{-9} M) reduced cell growth at a lower confidence level ($p<0.05$). In

contrast, treatment with analog V (10^{-9} M) gave a stronger anti-involucrin reactive band, with no significant inhibition of cell growth.

The applicants have therefore shown that vitamin D analogs inhibit proliferation and promote differentiation in canine cancer cells. Parenteral administration of vitamin D analogs to pets would necessitate the involvement of a veterinarian, which would substantially increase the expense. The applicants have found, however, that enteral administration of vitamin D analogs is also effective for cancer therapy in dogs, and that incorporating vitamin D analogs into dog food is an effective and practical way of routinely administering vitamin D analogs to a pet suffering from cancer. In practice, the therapeutic efficacy against cancer of a pet food containing vitamin D analog is evaluated by methods well-known to those skilled in the relevant art. For example Valierus *et al.* detail methodology used for evaluating a variety of anti-cancer therapies, the entire disclosure of which is hereby incorporated by reference (Valerius *et al* 1997).

The vitamin D analog incorporated into dog food can be processed in accordance with conventional methods to produce pharmaceutical agents for administration to patients, e.g., in admixtures with conventional excipients such as pharmaceutically acceptable organic or inorganic carrier substances suitable for oral administration that do not deleteriously react with the active compounds. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt (buffer) solutions, alcohols, gum arabic, mineral and vegetable oils, benzyl alcohols, polyethylene glycols, gelatine, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, and polyvinyl pyrrolidone. The pharmaceutical preparations can be mixed, if desired, with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic active compounds. The dosage forms may also contain adjuvants, such as preserving or stabilizing adjuvants. They may also contain other therapeutically valuable substances or may contain more than one of the compounds specified herein and in the claims in admixture.

In general, the daily dosage of the compounds according to this invention generally is about 0.025 to about 500 nmol/kg of body weight of the patient, and preferably about 0.025 to about 100 nmol/kg. In a more preferred embodiment, the daily dosage is about 0.025 to about 10 nmol/kg of body weight of the patient, and in a most preferred embodiment the daily dosage is from about 0.025 to about 1 nmol/kg of body weight of the patient.

In addition, those skilled in the art will also appreciate that such dosages may be encapsulated in time release, e.g., sustained, delayed or directed release delivery systems such as a liposome delivery system, polysaccharides exhibiting a slow release mechanism, salistic or other polymer implants or microspheres, as well as those where the active ingredient is suitably protected with one or more differentially degradable coatings, e.g., by microencapsulation, enteric coating, multiple coatings, etc., and such means effect continual dosing of compositions contained therein. For example, an enteric coating is suitably one which is resistant to disintegration in gastric juice.

It will be appreciated that the actual preferred amounts of active analog in a specific case will vary according to the specific compound being used, the particular compositions formulated, the mode of application, and the particular sites being treated. Dosages can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compounds and of a known agent, e.g., by means of an appropriate conventional pharmacological protocol.

The specific doses for each particular patient depend on a wide variety of factors, for example, on the efficacy of the specific compound employed, on the age, body weight, general state of health, sex of patient, on the diet, on the timing and mode of administration, on the rate of excretion, and on medicaments used in combination and the severity of the particular disorder to which the therapy is applied.

The dosage forms may also contain adjuvants as well as other therapeutically valuable substances or may contain more than one of the compounds specified herein in admixture. Thus, a further aspect within the scope of the present invention is administration of effective dosages of the compounds of the present invention in conjunction with administration of other hormones or other agents that

have been shown to have efficacy in the treatment and present of the diseases and disorders described herein.

For example, compounds of the present invention are suitably co-administered with agents known to ameliorate bone diseases or disorders. Such bone agents may include conjugated estrogens or their equivalents, antiestrogens, calcitonin, bisphosphonates, calcium supplements, calcium receptor agonists, cobalamin, pertussis toxin, boron, dehydroepiandrosterone (DHEA) and other bone growth factors such as transforming growth factor beta, activin or bone morphogenic protein.

Also provided herein are compounds of the present invention that are co-administered with known cytotoxic agents. Such agents include estramustine phosphate, prednimustine, cisplatin, 5-fluoro-uracil, melphalan, hydroxyurea, mitomycin, idarubicin, methotrexate, adriamycin, daunomycin, cyclophosphamide, doxorubicin (hydroxydaunorubicin), vincristine (oncovin) and pregnisone. It is anticipated that a 1α-hydroxyvitamin D of the present invention used in combination with various anticancer drugs can give rise to a significantly enhanced cytotoxic effect on cancerous cells, thus providing an increased therapeutic effect. Specifically, as a significantly increased growth-inhibitory effect is obtained with the above-disclosed combinations utilizing lower concentrations of the anticancer drugs compared to the treatment regimens in which the drugs are used alone, there is the potential to provide therapy wherein adverse side effects associated with the anticancer drugs are considerably reduced than normally observed with the anticancer drugs used alone in larger doses. Possible dose ranges of these co-administered second anticancer agents are about 0.1 μg to 1 μg/kg/day.

The compounds in accordance with the present invention are also suitably co-administered with known antiinflammatory agents. Such agents include both steroidal (e.g., corticosteroids) and nonsteroidal antiinflammatory agents (e.g., salicylates, naproxen). It is anticipated that a compound of the present invention used in combination with these various anti-inflammatory drugs can give rise to a significantly enhanced anti-inflammatory activity, thus providing an increased therapeutic effect.

For treatment purposes, the active compounds of this invention can be formulated as solutions in innocuous solvents, or as emulsions, suspensions or dispersions in suitable innocuous solvents or carriers, or as pills, tablets or capsules, containing solid carriers according to conventional methods known in the art. Any such formulations may also contain other pharmaceutically-acceptable and non-toxic excipients such as stabilizers, anti-oxidants, binders, coloring agents or emulsifying or taste-modifying agents.

The present invention is further explained by the following examples, which should not be construed by way of limiting the scope of the present invention.

EXAMPLES

Materials and Methods

Vitamin D and its analogs. A 1 mM stock solution of $1,25(OH)_2D_3$ and each of its analogs in absolute ethanol was prepared and protected from light. Maximum concentration of ethanol in the culture ($\leq$ 0.1%) did not influence cell growth or differentiation. Stock solutions of each compound were made in ethanol and Williams' E media (W ME) to concentrations of 10^{-7} M, 10^{-9} M, and 10-11 M just prior to culture.

Cell culture. SCC 2/88 cells were grown in W ME supplemented with 10% fetal bovine serum, 50 μg/mL of gentamicin, 10 ng/mL of epidermal growth factor (Gibco BRL, Grand Island, NY), 0.1 nM Cholera toxin (Calbiochem, La Jolla, CA), and 2 mM L-glutamine (Gibco BRL, Grand Island, NY) at 37°C, 5% CO_2, humidified atmosphere. Cells were seeded at a density of 10^5 cells/well in 6-well culture plates (Becton Dickinson, Franklin Lakes, NJ) and grown for 24 hours before starting experiments (day 0). After a 24-hour incubation at 37°C, medium containing vehicle (ethanol), $1,25(OH)_2D_3$, or its analogs (EB 1089 and analog V) was added at 10^{-7} M, 10^{-9} M, and 10^{-11} M and was changed every day for up to 3 days. Each experiment was run in triplicate. Media were collected every 24 hours for 3 days and stored at –70°C until assayed for PTHrP content by immunoradiometric assay. At the end of day 3, cells were retrieved from the 6-well culture plates by use of 250 μl GITC (4 M guanidine isothiocyanate, 0.5% sarcocyl, 25mM sodium citrate) per well

and stored at -70°C until assayed for cell proliferation by fluorescence DNA concentration analysis.

For RNA isolation, SCC 2/88 cells (2×10^6 cells/mL) were seeded in 90-mm tissue culture dishes (Becton Dickinson, Franklin Lakes, NJ) and grown up to 70% confluence in W ME media containing 10% FBS. Cells were incubated in the media without FBS for 24 hours before the time of treatment. Cells were treated with 10^{-7} M $1,25(OH)_2D_3$, 1.5 ng/mL of TGFβ, and 5 μg/mL of anti-TGFβ for 0, 3, 6, 12 hours. Cells were washed (phosphate buffered saline), trypsinized, and stored at -70°C until assayed by northern blot analysis.

Fluorescence DNA concentration analysis. DNA content of cell lysates was determined by DNA fluorometry through use of a fluorescent plate reader and analyzer (IDEXX Laboratories Inc., Westbrook, ME) and Hoechst 33258 dye (Hoefer Scientific Instruments, San Francisco, CA). Calf thymus DNA (100 μg/mL) served as a calibration control.

Total RNA isolation and Northern blot analysis. Total RNA was isolated by use of a Purescript® RNA isolation kit (Gentra systems, Minneapolis, MN) according to the procedures recommended by the manufacturer. Equal amounts of each RNA sample (20 μg loaded in each lane) were separated on a 1.2% agarose-formaldehyde gel, and transferred to a nylon membrane (Poll Biosupport, East Hills, NY). Northern blotting was conducted using standard procedures (Sambrook et al. 1989). Blot was hybridized with a ^{32}P-labeled cDNA probe (NEN life science products, Inc. Boston, MA). The nylon membrane was washed twice with a solution of 2× standard saline citrate buffer and 0.1 %(w/v) sodium dodecyl sulfate at room temperature for 15 minutes, then washed once with a solution of 0.1×standard saline citrate buffer and 0.1 %(w/v) sodium dodecyl sulfate for 30 minutes at 60°C for a high-stringency wash. Subsequently, the membrane was exposed through use of a phosphoimager screen. After exposure, the membrane was stripped and hybridized with a glyceraldehyde 3-phosphate dehydrogenase cDNA probe to normalize for RNA loading.

PTHrP immunoradiometric assay. Medium (200 μl) collected from cells treated with vehicle, $1,25(OH)_2D_3$, or its analogs (at concentrations of 10^{-7} M and

10^{-9} M) on days 0, 1, 2, and 3 in triplicate was assayed for PTHrP content. PTHrP was measured using immunoradiometric assay kit (DiaSorin Corp., Stillwater, MN) with human recombinant PTHrP 1-84 for standards and controls. Immunoradiometric assay was performed by binding of anti-PTHrP 1-40 antibody to polystyrene beads and labeling of the anti-PTHrP 57-80 antibody with 125I. Samples were incubated with the antibodies, and the polystyrene beads were then washed to remove any unbound-labeled antibody. The radioactivity remaining from the bound-labeled antibody was measured with a gamma-radiation counter. PTHrP content was quantified through use of a GraphPad Prism™ program (GraphPad software Inc., San Diego, CA).

Cell pellet procedure for immunohistochemical staining. SCC 2/88 cells were grown to approximately 8-9×10^6 cells on 10-mm tissue culture dishes. Cells were trypsinized and centrifuged at 3000 G, 4°C for 10 minutes. Supernatant was suctioned off to leave the cell pellet. Dissolved agarose was added to hold cells together. Immediately, the pellet was fixed in 2-methylbutane in liquid nitrogen for 20 seconds, and then was placed in the fixative (0.5% glutaraldehyde in absolute ethanol) overnight at –70°C. The pellet was dehydrated in absolute ethanol for an hour and in acetone twice for 30 minutes, respectively. The pellet was embedded and cut at 5 μm for immunohistochemical evaluation.

Immunohistochemistry. Staining for vitamin D receptor distribution was performed by incubating respectively with 5% normal goat serum in phosphate buffered saline (pH 7.4) for 30 minutes, primary antibody–rat monoclonal antibody against the vitamin D receptor (Chemicon International Inc., Temecula, CA) 1:50 at 4°C overnight, secondary antibody–goat anti-rat IgG (Chemicon International Inc., Temecula, CA) 1:20 in phosphate buffered saline for 30 minutes, rat peroxidase-anti-peroxidase (PAP) (Chemicon International Inc., Temecula, CA) 1:100 in 1% normal goat serum in phosphate buffered saline for 30 minutes, and 0.05% diaminobenzidine and 0.01% hydrogen peroxide in 0.05 M Tris buffer for 5 minutes. Slides were washed between each step with phosphate buffered saline, then dehydrated, mounted with aqua- Mount and visualized by light microscopy.

For PTHrP distribution the staining was done by blocking with 3% H_2O_2 for 20 minutes and incubating respectively with 2% normal horse serum in

phosphate buffered saline for 20 minutes, primary antibody – rabbit anti-human PTHrP (Oncogene research products, Cambridge, MA) 1:100 in primary antibody diluent at 4°C overnight, secondary antibody-biotinylated goat anti-rabbit IgG (Calbiochem, La Jolla, CA)) 1:500 in phosphate buffered saline for an hour, avidin-biotin complex (Pierce, Rockford, IL) for 30 minutes, and diaminobenzidine for 5 minutes. Slides were washed between each step with phosphate buffered saline and distilled water, then dehydrated, mounted with aqua- Mount and visualized by light microscopy.

Cell differentiation. Involucrin was extracted from cultured SCC 2/88 cells grown in 175 cm2 culture flasks (Becton Dickinson, Franklin Lakes, NJ) for one week. Post confluent cells were treated with either vehicle, $1,25(OH)_2D_3$, EB 1089, or analog V at concentrations 10^{-7} M and 10^{-9} M for up to 7 days. Cells were washed and released from the culture flask with phosphate buffered saline containing 20 mM EDTA. Cells were disrupted with a Branson sonifier (Branson Ultrasonic Corporation, Danbury, CT) at a setting of 6 for 3 × 30 seconds and centrifuged at 100,000 G for 30 minutes at 10°C. The supernatant (cytosol) was made 10% in glycerol and 62.5 mM in Tris-HCl (pH 6.8) and heated for 10 minutes at 100°C. The denatured proteins were removed by centrifugation at 15,000 G for 15 minutes. Involucrin in the supernatant was collected stored at -70°C until Western blot analysis was performed.

Western blot analysis. Extracted proteins (20 μg) were separated by electrophoresis through 7.5 % sodium dodecyl sulfate (SDS)-polyacrylamide gel, and transferred to nitrocellulose membrane with use of a semi-dry transferred technique (Bio-Rad laboratories, Hercules, CA). The membrane was then blocked in blocking solution (10% dry milk, 0.05% Tween 20 in phosphate buffered saline) overnight at 4°C. The preblocked membrane was incubated in mouse monoclonal antibody against involucrin (Research Diagnotics Inc., Flanders, NJ) 1:500 for an hour and extensively washed in PBS (pH 7.4) containing 0.05% Tween 20. The blot was then incubated in goat anti-mouse IgG (horseradish peroxidase) (Bio-Rad laboratories, Hercules, CA) 1:500 for an hour. After further washing in phosphate buffered saline containing 0.05% Tween 20, the blot was developed for 1 minute in the LumiGLO Chemiluminescent substrate (Kirkegaard and Perry laboratories, Gaithersburg, MD),

and then exposed to x-ray film for 1-5 seconds. After exposure, the membrane was stripped and incubated with mouse monoclonal anti-β-actin (Sigma, Saint Louis, MO) to normalize for protein loading. AlphaImager™ Alpha Innotech Corporation, San Leandro) measured the density of involucrin bands.

Statistical Analysis. Numerical data from PTHrP production and DNA concentration studies were analyzed by one-way analysis of variance (ANOVA), and Turkey's multiple comparisons test. Data from PTHrP (pg) per DNA (μg) studies were analyzed by t-test and ANOVA. The level of significance were established at $p<0.05$, $p<0.01$, or $p<0.001$ using Instat program (Graph PAD software Inc., San Diego, CA). The results were expressed as the mean ± standard error of the mean (SE M) (n = 3). All treatment groups were tested in triplicate.

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